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IMMUNITY IN DENGUE (U)

ANNUAL AND FINAL REPORT

by

Scott B. Halstead, M.D.

1 September 1983

(For the period 1 June 1969 to 31 December 1982)

Supported by

U.S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DADA17-69-C-9146
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University of Hawaii
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Uncloned dengue (DEN) 4 (H-241) passaged 15, 30 and 50 times in primary dog kidney (PDK) cells were subjected to two successive terminal dilution procedures. In the first (3C1), virus was diluted at 10-fold steps in 10 replicate tubes. A dilution row with three or fewer virus-infected tubes was selected for two further passages. In the second (TD3), virus was triple terminal diluted using 2-fold dilution steps and selecting one positive tube out of ten. Both procedures selected virus populations which differed from antecedents. Plaque size of PDK 15 was medium, PDK 30, small and PDK 50, pin-point. PDK 19-3C1 and 34-3C1 were medium and 56-3C1 small; 24-TD3, 35-TD3 and 61-TD3 were all small. All cloned viruses exhibited complete replication shut-off at 38.5°C, while PDK 15 and 30 had not. Cloned viruses were either avirulent for monkeys (19-3C1, 56-3C1, 24-TD3 and 35-TD3) or produced revertant large plaque parental-type viremia (34-3C1 and 61-TD3) compared with uncloned viruses which showed a graduated decrease in monkey virulence with PDK passage.

Two strains of primary dog kidney passaged dengue (DEN) 4 (H-241) virus which had been cloned by terminal dilution (PDK 24-TD3 and 35-TD3) were propagated in fetal rhesus lung (FRhL) cells preparatory to virus seed production. Both serial passage and prolonged replication of PDK 24-TD3 in FRhL resulted in appearance of medium and large plaques. When picked these were temperature-resistant monkey-virulent revertants. Serial passage and prolonged replication of PDK 24-TD3 in LLC-MK2 did not result in reversion; but, prolonged replication in PDK did. Passage of PDK 35-TD3 in FRhL cells resulted in appearance of medium size plaques. When picked, these viruses retained ts and low monkey virulence. Because of its stability in FRhL cells, reduced monkey virulence and ts properties, PDK 35-TD3 is a promising candidate for trial in man. Accordingly, this virus was propagated in large volumes. Production seed (FRhL p2) and Candidate vaccine (FRhL p3) were subjected to rigorous safety tests which excluded contaminating microbial agents. There was no significant monkey neurovirulence of parental or PDK passaged DEN-4 virus or by control fluid cultures. FRhL-passaged viruses retained phenotypic characters of small (occasional medium) plaque, ts at 38.5°C, no plaque formation in GMK, cytopathic effect in LLC-MK2 or viral growth in human monocytes. FRhL p2 virus displayed low virulence for monkeys; only one of four animals was viremic and three of four developed low titered antibody. FRhL p3 virus produced viremia in three monkeys and moderate to high hemagglutination-inhibition and neutralizing antibody titers in all animals. Virus at both passages in FRhL exhibited reduced neurovirulence in suckling mice as compared to parental DEN-4.

Because of its safety and desirable monkey virulence attributes PDK 35-TD3 FRhL p3 is recommended for human phase I trial.

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Prototype dengue (DEN) 4 (H-241) was received as original viremic human serum and passed once in a susceptible monkey and twice in Aedes albopictus replicated in primary dog kidney and African green monkey kidney cells and characterized at passages 7, 15, 30 and 50. Serial weekly passage of undiluted virus was carried to the 50th passage in both primary cell cultures. Parental DEN-4 phenotype included large plaque formation in LLC-MK2 cells, plaque formation in GMK cells, cytopathic effect in LLC-MK2, growth in human monocyte

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SUMMARY

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Because of its safety and desirable monkey virulence attributes PDK 35-TD3 FRhL p3 is recommended for human phase I trial.

FOREWORD

Sidney Gaines, Ph.D. and John James, Ph.D. have served as Quality Assurance Officers.

Virus attenuation studies have been conducted with professional assistance of Drs. Nyven J. Marchette, Arwin Diwan, Nicholas Palumbo and the technical assistance of Ms. Ravithat Putvatana, Kay Larsen, Laddawan Srisukonth, C.N. Venkateshan, Susan Kihara, Karen Shigematsu, Helen Sullivan, Clement Lopez, Kannan Eluthesen, Eddie Lau, Paige Awai, Sheile Kawamoto, Karen Amii and Ann Cutting. Numerous student workers assisted in the care and handling of rhesus monkeys, preparation of media and washing and sterilization of glassware.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. Statement of the problem and background.

Dengue viruses of four types have been the cause of significant morbidity in U.S. military personnel. Two distinct syndromes are known: dengue fever, a temporarily incapacitating self-limited febrile illness; and dengue hemorrhagic fever, a severe disease with appreciable mortality. Because the control of dengue vectors is not effective in most tropical areas, immunization may be the only practicable way to protect U.S. personnel on duty in these areas. At present, areas of dengue endemicity include the Caribbean basin, West Africa, the Indian subcontinent, Southeast Asia and many Western Pacific islands. Although monovalent vaccines might be useful under defined epidemiological circumstances, to provide for any contingency, a method of producing immunity to all four dengue types is needed. Our earlier studies in monkeys showed that infection with three or all four dengue virus types can be administered simultaneously and induce solid immunity.[1] This provides the basis for the proposition that under appropriate circumstances a live attenuated vaccine containing all dengue virus types may usefully protect humans against dengue diseases.

II. Results

A. Materials and Methods

1. Viruses

For certain preliminary studies the following dengue strains were used: DEN-1 (16007), DEN-2 (16681), DEN-3 (16565) and DEN-4 (4328S) (Halstead prototype). These strains of Southeast Asian origin have been propagated in tissue culture since recovery from infected humans.[2]

2. Parental DEN-4

Dengue 4 viruses, H-241 strain, was recovered from a serum obtained in Manila, Philippines in August 1956 from a child hospitalized with clinical illness symptoms compatible with dengue fever.[3] Original viremic serum from this patient was obtained by Dr. Leon Rosen from Dr. William McD. Hammon in 1969. On 15 August 1969, in Rosen's laboratory, this serum was inoculated into a susceptible Macaca irus monkey. Viremic monkey plasma was stored at -70°C. On 4 October 1971, 4 November 1971 and 18 January 1972, three serial intrathoracic passages were made in adult Aedes albopictus starting from viremic monkey serum. A saline suspension of third-passage triturated mosquito tissues was used as starting material for inoculation of tissue culrures for vaccine development attempts. For parental virus biological marker studies, tests were done on this virus at LLC-MK2 passage two or three.

3. Virus Assay

A single agar overlay plaque assay on LLC-MK2 monolayers[4] was used with some modivications.[2] LLC-MK2 cells were propagated in 10% calf

serum and Eagles Basal Medium (BME) with Hanks' buffered salts (HBSS), while the agar overlay consisted of a final concentration of 1% Noble agar, 10% heat-inactivated calf serum in BME with Earle's salt solution (EBSS). LLC-MK2 cells were propagated in one ounce prescription bottles. Monolayers were incubated with 0.2 ml virus suspension for 60 minutes at 37°C. Following the addition of agar overlay medium, cells were incubated in the dark for 7 days at 37°C, then for an additional 7-14 days at room temperature during which time plaque size enlargement and plaque definition were observed. Plaque size was scored after 7 days at room temperature.

4. Plaque Picking

Virus from distinctly separated plaques was transferred by inserting a sterile pasteur pipet below the agar, expressing approximately 0.2-0.3 ml maintenance medium which was then re-aspirated, and either stored at -70°C or inoculated on LLC-MK2 cells for amplification.

5. Cell Cultures

Primary Rabbit Kidney (RK). Cells were obrained from M.A. Bioproducts, Bethesda, Maryland. They were grown in 10% fetal calf serum (FCS) in BME, HBSS and maintained in BME-HBSS with 2% FCS.

Primary Duck Embryo (DE). Cells were obtained from M.A. Bioproducts, grown on BME, Hanks' BSS with 5% FCS and maintaned on BME, Earles's BSS with 2% FCS.

WI-38. Cells were obtained from American Type Culture Collection, grown and maintained on MEM, EBSS with 10% fetal calf serum.

Primary African Green Monkey Kidney (GMK). Kidneys were obtained from four young adult African green monkeys. These animals were housed individually in cages with closed sides. For 9 months animals were kept in separate rooms and precautions taken to prevent cross-infection from humans or animals of other species. Monkeys were tested with tuberculin at no less than 3 month intervals and two weeks prior to removal of kidneys. Animals were biochemically normal during the period of quarantine. No parasites were detected in blood or stool. At post-mortem, animals were found normal on gross and histological examination.

Cells were trypsinized and grown in BME, HBSS with 10% fetal calf serum and maintained in BME Earle's BSS with 2% fetal calf serum.

Primary Dog Kidney (PDK). Cells were obtained from Hawaii-quarantined beagles or from beagle puppies from the pathogen-free colony, Dow Chemical Company, Indianapolis, Indiana. Cells were grown and maintained on the same media used for GMK. All PDK cells were propagated on a single lot of fetal calf serum, lot number 45533, Flow Laboratories, Rockville, Maryland.

In Hawaii two adult beagles were used which had been maintained for four months in isolated cages in vermin-free quarters at the Quarantine Station, Department of Agriculture, State of Hawaii. These dogs were in

overt and good health, had no direct exposure to other dogs or animals during the quarantine period. They were quarantined for an additional 6 month period in air conditioned quarters at the University of Hawaii at which time their blood chemistries were found to be normal and they were found free of canine parasites. The animals were clinically free of tuberculosis, infectious canine hepatitis, canine distemper, rabies and leptospirosis. After removal of both kidneys, dogs were autopsied by a veterinary pathologist. By gross and histological examination both animals were free of any disease.

Primary Chick Embryo (CE). CE monolayers were prepared by the method of Dulbecco.[4] Cells were grown in BME, EBSS supplemented with 2% heat-inactivated chicken serum and 2% tryptose phosphate broth.

Fetal Rhesus Lung Cells. Fetal rhesus lung cells designated DBS-FRhL-2 were received from the Walter Reed Army Institute of Reseerch either as passages 17 or 18. The origin of these cells has been described.[5] FRhL cells were grown in 10% fetal calf serum (FCS), minimal essential medium (MEM), Earle's buffered salt solution (EBSS), glutamine, bicarbonate and antibiotics and maintained in the same emdium using 2% FCS.

6. Release of Virus from Cells Using $MgSO_4$

Because of the consistent low release of PDK replicated virus into extracellular fluids from infected FRhL, alkali salts were edded to harvest media as described by Matsumura and Schlesinger.[6] From preliminary tests on PDK 61-TD3-infected PDK and FRhL cells trested with various concentrations of $MgSO_4$, $MgCl_2$ and $NaCl_2$ an optimal relcase medium was selected. Sodium chloride effected an 10-100-fold increase in extracellular virus as early as 204 hours after infection with no difference over an applied concentration range of 10-70 mM. Magnesium chloride at concentrations ebove 30 mM produced 100-1000-fold increase in cells infected for 192 hours. Magnesium sulfate produced the greatest effect over a wide range of concentrations (10-70 mM). After 204 hours infection, greater than 1000-fold release was effected in 15 minutes. Over a period of 96 hours et 8 to 12 hour intervals, pulses of 70 mM $MgSO_4$ resulted in substantislly the same release of virus into maintenance medium.

The following procedure was used for preparation of large seed pools (Master Seed, Production Seed) of cloned DEN-4 viruses: after 7 days incubation, maintenance medium wes removed from infected FRhL cells, pooled snd seved at -70°C. For one-ounce prescription bottles, 4 ml of a medium consisting of 3.5% human albumin, MEM, entibiotics and e finel concentration of 70 mM $MgSO_4$ was edded to cells. After 30 minutes this medium was removed, pooled and saved et -70°C. Normal maintenance medium was replaced, cells incubated at 32°C and this procedure repested on days 8, 9 and 10. Sublots containing the highest titers were pooled as seed viruses.

7. Frozen Storage of Cells

Following primary trypsinization, cells were grown to confluency in 32 ounce glass bottles. Cells monolayers were washed with PBS, trypsinized, then washed twice with BME-HBSS snd cell concentrations sdjusted to 3×10^6 cells/ml in BME-EBSS with 10% FCS and 10% glycerol. One ml smounts were

placed in glass vials, sealed and then frozen in styrofoam boxes held in an ultra-low temperature freezer (-70°C). Frozen cells were transferred to liquid nitrogen.

8. Safety Tests on Cells and Culture Media

After each primary trypsinization monolayers were grown in ten screw-capped test tubes. When fully confluent the medium was changed to maintenance medium. These tubes (Lot control) were observed microscopically every day for 14 days. On the 5th day, medium from each tube was removed, pooled and stored at -70°C. Culture fluids were tested in Sabouraud's medium, thioglycollate and blood agar. To test for mycoplasma, fluids were inoculated into PPLO enrichment broth (Difco) for 48 hours. Enriched broth was plated in duplicate on mycoplasma agar. One set of plates was incubated aerobically and one set anaerobically at 37°C. Unknowns and positive control plates were observed for mycoplasma colonies under the microscope. On the 14th day, Dienes stain was added to culrures. They were sub-inoculated into WI 38, GMK, PDK and RK tubes. On the 14th day, medium was removed and 0.5 ml of 0.2% fresh, saline-washed guinea pig erythrocytes (RBC) added to each tube. Tubes were kept at room temperature for 30 minutes and observed microscopically for hemadsorption.

9. Temperature Sensitivity Studies

Temperature of replicative shut-off was measured using two methods:

a) Growth curve method. Virus was added to LLC-MK2 monolayers in screw-capped test tubes which were incubated in a circulation-type water bath accurate to $\pm$ 0.1°C. Temperatures were monitored two times per day using a calibrated mercury thermometer. Replicate infected tubes were removed at intervals as designated in the text. Virus content of cells and supernatant fluids were quantitated separately or combined. Control virus infected cultures were incubated in an incubator at 37°C, assayed and growth curves compared.

b) Efficiency of plating method. LLC-MK2 monolayers in rubber stoppered one-ounce prescription bottles were inoculated with serial $\log_{10}$ virus dilutions, agar overlay added and placed in a water-tight box, weighted and immersed at a specified temperature in a circulating water bath for 7 days. Replicate plaque assay cultures were held at 37°C in an incubator for seven days. Plaque forming units (PFU) per ml of original virus seed were compared for the different temperatures tested.

10. Monkey Neurovirulence

Healthy rhesus monkeys were free of neutralizing antibodies to DEN 1-4 viruses. Animals were weighed prior to inoculation. Under deep penthothal anaesthesia 0.1 ml undiluted virus was inoculated into thalamus bilaterally, 0.1 ml of undiluted virus into lumbar spinal cord enlargement L1-L2 and 0.1 ml undiluted virus was inoculated into the quadriceps muscle bilaterally. Monkeys were weighed daily, rectal temperatures were taken

daily and animals were observed for signs of paralysis or changes in behavior. After 17 days, animals were sacrificed and samples of fresh tissue taken from cerebral cortex, thalamus, cervical and lumbar spinal cord for virus isolation. Tissues were fixed in 10% biffered formalin, histological sections were prepared and examined for evidence of viral cellular damage.[7]

11. Antibody Tests

Antibody tests were done using lightly heparinized plasma (20 IU/ml final concentration). Non-specific inhibitors were removed with Kaolin, RBC agglutinins removed with goose RBC and plasmas tested for hemagglutination-inhibition in microvolumes using standard methods.[8] Plasmas were tested at an initial dilution of 1:20 versus 4-8 units of hemagglutinin. Neutralizing antibodies were tested at the 50% plaque reduction end point using the single agar overlay LLC-MK2 assay.[9] Serial four-fold dilutions were tested from an inital dilution of 1:10 versus 30-50 plaque forming units (PFU).

12. Separation of Immunoglubulins

IgG and IgM were separated by sucrose density gradient ultracentrifugation. Individual fractions of 0.05 of 0.1 ml were tested for antibody and tested for Ig isotype by Ouchterlony method using commercial reagents.

13. Suckling Mouse Neurovirulence

One hundred PFU of test strain and parental virus were inoculated intracerebrally in 24 hour-old mice. Mice were observed for deaths daily to 21 days and average survival times calculated. For the purposes of this calculation, mice surviving over 21 days were scored as dying on day 21.

14. Intrathoracic Inoculation of Aedes albopictus

Adult male Aedes albopictus were inoculated with virus preparations intrathoracically as described by Rosen and Gubler.[10] Mosquitoes were held at 28°C for 10 days, triturated and tested for virus content by LLC-MK2 plaque assay.

15. Growth of Virus in Antibody Supplemented Human Monocyte Cultures

Mononuclear leukocytes were separated from whole blood on ficoll-hypaque[11] and cultured in RPMI medium supplemented with 10% FCS, bicarbonate, antibiotics and glutamine. Virus at a multiplicity of infection (MOI) of approximately 0.01 was added to cells cultured at 1×10^6/ml. Cultures received monkey anti-DEN-3 at a final dilution of 1:500. Cultures were incubated at 37°C. Virus yields were assayed on days 3, 4 and 5.

16. Terminal Dilution Methods

Viruses were cloned using serial 10-fold and then serial 2-fold dilutions successively. PDK cells were propagated in screw-capped test tubes. Ten tubes were individually inoculated with virus dilutions. Each tube was assayed on LLC-MK2 monolayers and virus-containing tubes selected for sub-passage when not more than three of ten was positive (10-fold dilution series) or only one of ten was positive (2-fold dilution series). Sub-passage tubes were inoculated in 10- or 2-fold dilution steps and the procedure repeated a total of three times.

B. Results

1. Uncloned Virus

a. Permissiveness of selected primary cell cultures to dengue virus replication

To test the ability of dengue 1-4 viruses to grow in cells used in licensed attenuated viral vaccines, inocula of Halstead prototypes were diluted to produce an approximate multiplicity of infection (MOI) of 0.1. After 90 minutes adsorption at 37°C, cell sheets were washed 3 times, 1.0 ml maintenance medium added and tubes incubated at 37°C. Over a period of two weeks, at daily intervals, tubes were harvested and growth curves constructed by titrating a mixture of freeze-disrupted cells and extracellular fluid in the LLC-MK2 plaque system. All four DEN viruses replicated to reasonably high titer in PGMK (circa 10^4-10^5 PFU/0.2 ml). Moderate growth (circa 10^2-10^3 PFU/0.2 ml) occurred with all four DEN viruses in PDK cells at 37°C. No replication of any DEN virus was observed in CE, DE or RK cells. Growth of DEN 1-4 viruses in PDK was studied further at 32°C. Figure 1 shows good growth by all four dengue types.

b. Serial passage of DEN-4 (H-241) in PDK and GMK

Thermal degradation curves at 32°C of DEN-4 (4328S) in cell-free PDK maintenance medium demonstrated persistance of virus until but not after 72 hours. With this evidence and using growth curve data, it was decided to serially passage wild-type DEN-4 (H-241) in PDK and GMK cells at weekly intervals. Passage history is summarized in Table 1. All inocula into screw-capped test tubes were 0.1 ml of undiluted virus. At passages 7, 15, 30 and 50, derivative strains were characteriized for various biological markers (Tables 2 and 3).

c. Plaque morphology on LLC-MK2

A shift in plaque size appeared between the 7th and 15th passages both in GMK and PDK. By the 30th passage, PDK passaged virus produced uniform pinpoint plaques. GMK passaged virus produced small plaques with occasional large plaques at low dilutions.

d. Cytopathic effect in GMK and PDK cells

GMK cultures inoculated with serially passaged viruses were observed for CPE daily. The parental virus and all GMK passages produced no CPE in PDK or GMK cells. PDK adapted virus did not produce CPE in GMK or PDK cells at any passage level.

e. Temperature sensitivity

Temperature of replicative shut-off was measured at 37°, 38°, 39° and 40°C by the EOP method. PDK 50 showed 90-95% reduction in plaques at 39°C while DEN-4 parental exhibited 5-10% plaque reduction at this temperature compared with numbers at 37°C.

f. Growth in Aedes albopictus

All passage levels of DEN-4 in PDK and GMK (10^2-10^4 PFU) were inoculated intrathoracically in male Aedes albopictus. After 10 days at 28°C, mosquitoes were triturated and assayed for virus content (Table 4). Regardless of input dose, virus yields per mosquito were remarkably consistent except PDK 50 which showed a 1.5 log reduction in growth. Oral feeding was not attempted.

g. Monkey antibody response

A diminution in HI titer measured at day 42 after inoculation of susceptible rhesus monkeys was observed with both the PDK and GMK passage series (Table 5). The effect was noted at earlier passage level with the PDK line and was both more marked and more consistent at PDK 30 and above.

h. Monkey viremia

Parental DEN-4 (H-241) produced a consistent large plaque viremia pattern in susceptible rhesus monkeys. Viremia was detected either on days 1 or 2, usually lasting three days. HI antibody responses were consistently 1:160-1:640 in 14 animals tested (Table 5). In the PDK series, the percentage of monkeys demonstrating detectable viremia progressively diminished as PDK passage level increased (Table 6). With serial passage in GMK, one animal each at passage 30 and 50 experienced a large plaque viremia. When progeny from these plaques were replicated in LLC-MK2 and inoculated in a susceptible rhesus monkey, a wild-type viremia pattern was observed.

i. Challenge with parental dengue 4 virus

Six weeks following inoculation of PDK or GMK passaged DEN-4 viruses in susceptible rhesus monkeys, animals were challenged with 10^4-10^5 PFU of parental virus. Animals which had developed a low HI (1:10) or PRNT antibody titer to initial immunization responded to challenge with a marked secondary antibody response (Table 7). Animals with moderate antibody titers following initial infection demonstrated transient 2-4-fold

elevation in titers (usually on day 14) to parental challenge. Some monkeys which apparently failed to develop immunologic memory following inoculation (H-246, H-250), experienced a primary antibody response following challenge (Table 6, 7).

j. Suckling mouse neurovirulence

Over the range of passages in GMK DEN-4 showed no significant difference in mouse neurovirulence compared with parental virus (Table 2). However, a gradual lengthening of the average survival time was noted with progressive passage in PDK (Table 2). Virus recovered from mouse brain inoculated with PDK 50 had uniform pinpoint plaque morphology on LLC-MK2 cells. While a few large plaques were observed in mouse brain inoculated with GMK 50 virus. These were temperature resistnat and produced parental type viremia in monkeys.

k. Monkey neurovirulence

One juvenile rhesus monkey each was tested for neurovirulence with $2 \times 10^4 - 4 \times 10^5$ PFU of parental, PDK 15, 30 and 50 passages of DEN-4. Monkeys were clinically and biochemically normal during the 17 day observation period. On autopsy no gross anatomical lesions were noted which could be attributed to viral infection. Abnormal findings were limited to perivascular mononuclear cell infiltrates whose intensity decreased with higher PDK passage. No neuronal destruction was observed. No virus was recovered from any brain or spinal cord specimen tested. An observation of interest was that PDK 50 virus produced high titered HI antibody (1:640) after intracerebral inoculation. The same virus given by the subcutaneous route routinely railed to elicit detectable HI antibody response.

l. Safety tests and stability in human albumin

Each lot and batch of PDK and GMK were tested for bacterial, mycoplasmal and fungal contaminants and CPE producing or hemadsorbing agents. None were detected. Large lots of over 1000 ml of virus were prepared from PDK 15, 30 and 50 passage materials. These were stabilized in 3.5% human albumin. These suspensions have showed no significant titer loss over a ten year period at $-70^{\circ}C$.

2. Cloned Virus

In the preceding section the origin and biological attributes of parental H-241 strain of dengue 4 were described and compared with markers associated with virus which received different levels of serial passage in primary dog (PDK) and African green monkey (GMK) kidney cells. The most consistent change observed was a plaque size reduction which occurred in fewer than ten passages in both cell culture systems. With higher levels of PDK-passaged virus, modified viremia and antibody responses were observed following inoculation of susceptible rhesus monkeys. Over the same serial passage span in GMK, inconsistent changes in virus virulence for monkeys were seen. Other properties of viruses which received prolonged passage in

PDK cells were reduced growth at 39°C, reduced neurovirulence in suckling mice and reduced ability to replicate in human peripheral blood monocytes.

Because of the possible advantage that genetic purity might convey to marker stability with possible benefits to large scale vaccine production, a U.S. Army Scientific Advisory Committee recommended cloned selection of virus from different large lot PDK passaged virus. Inability to plaque PDK- or GMK-passaged DEN-4 virus in cells which are acceptable for vaccine development made it necessary to use terminal dilution method for clonal selection. Here we describe attributes of clonal populations selected by terminal dilution methods.

a. Initial cloning attempt

PDK 15, 30 and 50 viruses were terminally diluted three times using the 10-fold dilution method. This resulted in virus passage levels of 18, 33 and 53, respectively. Three strains are designated 3C1.

Characteristics of the selectants are given in Tables 8-10. All strains exhibited medium or small plaque formation on LLC-MK2 monolayers. PDK 19-3C1 and PDK 34-3C1 were 3 mm in size with hazy margins. PDK 56-3C1 plaques were pinpoint in size with sharp margins. Each of the 3C1 strains failed to replicate at 38.5°C, a temperature which supported replication of DEN-4 parent. The 3C1 strains shared the following attributes of their uncloned antecedents: failure to plaque in GMK cells, failure to produce CPE in LLC-MK2 cells and failure to replicate in antibody-supplemented cultures of human peripheral blood monocytes.

When studied, in vivo, one of the three 3C1 strains showed reversion to parental type compared with uncloned antecedent or the other two strains. Five of 6 susceptible rhesus monkeys developed viremia when inoculated with 400-1700 pfu of PDK 34-3C1. All viremia was large plaque which, when picked and replicated in LLC-MK2, exhibited wild-type temperature resistance. In contrast, inoculation of PDK 19-3C1 and PDK 56-3C1 failed to produce viremia in susceptible monkeys (Table 11). Detectable HI or neutralizing antibody responses were observed in only one half of monkeys inoculated with PDK 19 or 56-3C1 strains. When HI antibody responses were <1:20, challenge with 3.6×10^4 pfu of parental DEN-4 uniformly resulted in breakthrough viremia which was always accompanied by a secondary-type antibody response. One of six animals (H-191) inoculated with PDK 34-3C1 apparently failed to become infected. Following parental DEN-4 challenge this animal became viremic and had a typical primary antibody response.

b. Second cloning attempt

Because of the surmise that results described above might reflect incompletely cloned virus, the cloning technique was repeated, this time using 2-fold serial dilutions (TD3 series). Starting materials were PDK 19-3C1, PDK 30 (uncloned) and PDK 56-3C1. The scheme for selecting

clones is given in Figure 2. Three serial terminal dilutions selected viruses which produced very small plaques on LLC-MK2 cells. Occasional medium-sized plaques seen with PDK 24-TD3.

When TD3 clones were studied for biological markers, they were closely similar to one another and resembled PDK 19-3C1 and PDK 56-3C1 in exhibiting marked decrease in virulence for rhesus monkeys (Tables 9, 10, 12). Further details of viremia and antibody response to initial immunization and following challenge with parental DEN-4 are give in Table 13. Notable observations are that large plaque, temperature resistant virus was isolated from the blood of two of five monkeys inoculated with PDK 61-TD3 virus. All five animals developed either HI or neutralizing antibody following immunization with approximately 10^5 pfu; four were solidly protected from parental DEN-4 challenge.

3. The Problem of Reversion

In section 2., cloned virus, it was noted that six populations derived by terminal dilution of uncloned PDK 15, 30 and 50 all demonstrated small to pinpoint plaque formation and temperature sensitivity. Two clones, one derived from PDK 30 and the other from PDK 50, when passed into susceptible rhesus monkeys exhibited high frequency reversion. There remained two satisfactorily cloned viruses (PDK 24-TD3 and PDK 35-TD3): it remained to prepare large seed lots. For this the substrate FRhL was chosen.

Reversion was noted as a frequent problem.

a. Passage in LLC-MK2 cells

It was important to establish that the assay system did not by itself select for revertant virus. To do this cloned PDK viruses were serially passaged in LLC-MK2 cells. PDK 24-TD3, 35-TD3 and 61 TD3 viruses (average input doses of 1.4×10^4 pfu) were inoculated on LLC-MK2 monolayers. At seven days, infected LLC-MK2 and supernatant fluids were harvested and tested for plaque size and temperature sensitivity and further passaged two times. Although each of the three viruses showed a consistent increase in plaque size with successive passage, all retained $ts^{38.5}$ (Table 14).

b. Studies on PDK 24-TD3 serial passage in FRhL

PDK-TD3 viruses were passed ten times in FRhL. FRhL-2 cells were grown in 8 ounce prescription bottles. Each were inoculated with 100 pfu of DEN-4 virus, incubated at 35°C, harvested by three times freeze-thawing and then assayed for plaque size markers. PDK 24-TD3 retained small plaque size throughout ten passages. Small plaque PDK 24-TD3 FRhL p10 showed diminished growth, but not absolute shut-off at 38.5°C (Table 15).

c. Harvest after prolonged replication time in FRhL

PDK 24-TD3 was inoculated on monolayers of DBS-FRhL-2 grown to confluency in glass roller bottles. Cells were infected with

4×10^4 pfu and bottles rolled at 0.5 rpm at 35°C. Infected cells were pulsed with $MgSO_4$ as shown in Table 16. Virus was harvested at intervals over 19 days. On day 15 and 19 small numbers of large plaques were observed. When picked and replicated in LLC-MK2 the progeny were uniformly large plaque, temperature resistant and produced parental type viremia in susceptible monkeys. A similar experiment was done inoculating PDK 24-TD3 FRhL p1 into FRhL cells. In this experiment (FRhL p2) large plaques appeared as early as 12 days. In both experiments virus harvested before 12 days was uniformly pinpoint or small plaque, always ts and avirulent for monkeys.

Studies then were extended to PDK and LLC-MK2 cells. When PDK 24-TD3 was inoculated in PDK cells, as with FRhL cells, emergence of large parental type plaques required a minimum of 14 days (data not shown) but was independent of culture temperatures of 32°C and 37°C or of harvesting extracellular fluid with or without $MgSO_4$. In contrast, prolonged infection in LLC-MK2 did not result in plaque size change or ts of PDK 24-TD3 virus (Table 17).

When infectious inocula were themselves mixtures of small and large plaque viruses, the emergence of large plaques was independent of inoculum size (Table 5). However, substantially the same experiment done by passaging mixed small and large plaque PDK 24-TD3, FRhL p2 virus in PDK cells did not yield large plaque virus (Table 18). It should be noted that virus from infected PDK was harvested on day 9 or earlier than the time of appearance of large plaques in chronically infected PDK. However, when this small plaque PDK-derived virus was passed into FRhL cells, medium plaques which were temperature resistant appeared (Table 19).

d. Studies on PDK 35-TD3

Prolonged replication of PDK 35-TD3 in FRhL cells result in the emergence of medium plaques at 9 days. These were ts and monkey avirulent (Table 20). Serial passage of PDK 35-TD3 in FRhL cells also resulted in the appearance of medium plaques (Table 21). In this experiment, medium plaques did not appear at 7 days; however, medium plaques were consistently observed at 8 days and after (Table 22). Progeny from medium plaques exhibited replicative shut-off at 38.5°C.

A total of 40 individual medium and large plaques from FRhL passages 1 and 2 of PDK 35-TD3 were picked, replicated in LLC-MK2 cells and tested for replicative shut-off using the tube method. Each of these plaque picks was ts and produced homogeneous medium to large-size plaques. In no instance did a medium or large plaque virus produce pinpoint or small plaques.

Of 40 medium or large plaque picks studied for ts, 10 were inoculated in rhesus monkeys. Mixed plaque populations derived from virus replicated in FRhL were inoculated into 6 monkeys. All monkeys tested developed low to moderate titered HI antibody (20-80) and no or low levels of delayed viremia. The plaque morphology of virus recovered from blood

was a mixture of medium and large plaques (Table 23). Medium and large plaques were picked from virus recovered from 6 monkeys. All produced homogeneous medium and large plaque morphology and retained replicative shut-off at 38.5°C.

4. Preparation of Candidate DEN-4 Vaccine

The previous three sections have described biologic attributes of dengue (DEN) 4 H-241 strain serially passaged in primary dog kidney (PDK) or African green monkey kidney cells (section 1) or cloned by terminal dilution in PDK (section 2). Two of three cloned viruses produced parental-type revertants, one when replicated in PDK cells and one on passage into fetal rhesus lung (FRhL) cells (section 3). A third clone passaged in FRhL, PDK 35-TD3, produced plaque-size revertants but retained attributes of temperature sensitivity at 38.5°C and reduced virulence for susceptible rhesus monkeys (section 3).

Here is described the preparation, safety testing and biological characteristics of a candidate vaccine prepared from PDK 35-TD3 DEN-4 virus passaged three times in FRhL.

a. Production of seed virus and vaccine

Large lots of DEN-4 PDK 35-TD3 were prepared by initial passage in FRhL (Master Seed) and again a second passage in FRhL (Production Seed). Candidate vaccine was prepared from the third passage in FRhL. FRhL monolayers in 150 cm^2 flasks (Costar, Cambridge, Mass.) were inoculated with virus at a multiplicity of infection of approximately 0.005 and allowed to adsorb at 35°C for 1.5 hours. Maintenance medium consisted of Eagle's minimal essential medium with 2% fetal bovine serum (FBS), 0.22% $NaHCO_3$, streptomycin (50 µg/ml) and neomycin (100 µg/ml). On day 4 post-inoculation, all flasks were washed 3 times with 50 ml Hanks' balanced salt solution (HBSS) and re-fed with 50 ml maintenance medium containing 0.25% human serum albumin instead of FBS. On day 7 post-inoculation, supernatant fluids from DEN-4 infected flasks were replaced with 25 ml maintenance medium containing 70 mM $MgSO_4 \cdot 7\ H_2O$ (release medium). After incubation at 35°C for 30 minutes the supernatant fluids were removed and held at 4°C. The FRhL flasks were re-fed with 50 ml maintenance medium containing human serum albumin and no $MgSO_4$ supplement. On days 8, 9 and 10 post-inoculation the same procedure was used to harvest DEN-4 supernatant fluids. Each days harvest from each flask was held at 4°C until plaque assays indicated that yields were adequate and that the virus phenotype had not changed. Once this was established a pool of DEN-4 virus supernatant fluids was filtered using a 450 nm membrane filter (PALL Trinity Micro Corporation, Cortland, New York) and freeze-dried in 3 ml aliquots.

b. Safety testing

Tests for adventitious microbial agents in production seed and vaccine were performed following Public Health Service regulations for licensed, live attenuated virus vaccines (Code of Federal Regulations, Chapter 21, Subchapter F Biologics). The Production Seed and Candidate vaccine were produced at the Department of Biologics Research, Walter Reed

Army Institute of Research under conditions which satisfy Good Laboratory Practice Act regulations.

Neurovirulence studies were done on Production Seed, parental DEN-4 and virus-free cell culture fluids. Twenty-two mature rhesus monkeys, tuberculin negative and free of antibody hemagglutination-inhibition (HI) or plaque reduction neutralizing (PRNT) antibody against DEN-4 were anaesthetized using a combination of ketamine hydrochloride and pentothal, prepared for surgery and inoculated (0.5 ml) bilaterally into thalami and into the lumbar spinal cord at the L1-L2 intervertebral space. Three animals received virus-free cell culture fluids, 17 received candidate vaccine and two received parental DEN-4. Nineteen to 21 days after inoculation, monkeys were anaesthetized, euthanized, and perfused with 300-500 ml saline then 500-700 ml of 10% neutral buffered formalin. The entire brain and spinal cord was removed and immersed in formalin solution.. After at least 7 days immersion in formalin, sections were made of motor cortex, thalamus, mesencephalon, medulla oblongata, cervical spinal cord and lumbar spinal cord. Tissues were imbedded in paraffin, sectioned glass slide-mounted, stained with hematoxylin and eosin and examined microscopically.

c. Biological markers

Eleven of 24 harvest lots (100 ml each) of Master Seed produced only small plaques when assayed on LLC-MK2 cells. Five others produced an average of 1-11 medium or large plaques per one-ounce assay bottle. The lost without medium or large plaques were pooled and used as inoculum for Production seed. In turn, 16/24 harvest lots at FRhL passage 2 were free of medium or large plaques. Ten of these were pooled for Production Seed which was used to make candidate vaccine.

Fifteen separate aliquots of Production Seed and Candidate vaccine were separately assayed on LLC-MK2 cells. Five and four, respectively, contained 2-3 medium plaques per one-ounce bottle. One or more medium plaques were picked from each lot, assayed and tested for $^{ts}38.5$. Each showed no growth at this temperature.

Other in vitro biological markers were identical to those described for PDK 35-TD3 but differed from those of parental DEN-4 as summarized in Table 24. These markers are failure to plaque in GMK, failure to produce CPE in LLC-MK2 and failure to grow in cultures of human monocytes.

In susceptible rhesus monkeys, only one of four monkeys inoculated with FRhL p2 was viremic and that animal on a single day. Three of four of these animals developed HI and/or PRNT antibodies. And 2 of 4 responded to parental challenge with a secondary-type antibody response. All animals inoculated with FRhL p3 virus developed both HI and neutralizing antibodies; three were viremic. All four animals completely resisted parental virus challenge (Table 25).

The average survival time of 1-2 day old mice inoculated with 100 pfu of FRhL p2 and FRhL p3 viruses were significantly longer (15.5 and 16.9 days, respectively) than survival time following inoculation in the same groups of litters of parental DEN-4 (11.0 days). For these experiments 14, 15 and 16 mice were used, respectively.

No monkey inoculated with Production Seed, DEN-4 parent or virus-free control fluids demonstrated any abnormal neurological signs during 17-21 days observation period. Needle track lesions (NTL) were identified histologically in the thalamus in one animal receiving virus-free culture fluids, 7 receiving DEN-4 Production Seed, and one monkey receiving virus-free culture fluids. NTL were seen in lumbar spinal cord in all three animals receiving virus-free culture fluids, 7 receiving Production Seed and one monkey receiving parental DEN-4. Using a histological grading system based on intensity of cellular infiltrate, neuronal death and satellitosis, there was no evidence of neurovirulence in control monkeys. Seven of 17 animals receiving Production Seed had minimal grade 1 lesions in several brain or spinal cord sections; all monkeys receiving parental DEN-1 had minimal grade 1 lesions in a similar distribution. In none of the grade 1 lesions was there conclusive evidence of neuronal death, the predominant lesions being microgliosis, perivascular lymphocyte cuffing and occasional satellitosis.

It was concluded that neither the parental DEN-4 nor Production Seed exhibited significant viral neurovirulence.

III. Discussion and Conclusion

The studies on biological markers of dengue 4 candidate vaccine show satisfactory features to recommend PDK 35-TD3 for phase I human testing.

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Table 1. Passage history of uncloned DEN 4 (H-241) in PDK and GMK cells

Passage No.	Host	Identity No.	Date Inoculated	Day of Harvest	Comments
0	viremic serum	H-241	Aug 1956*	6	
1	Macaca irus	63	15 Aug 69	5	subcutaneous inoculation
1	Aedes albopictus	1030, 1031	4 Oct 71	10	intrathoracic passage
2	Aedes albopictus	2606	4 Nov 71	10	intrathoracic passage
3	Aedes albopictus	4943, 5000	18 Jan 72	10	intrathoracic passage
PDK					
1-5	PDK	I. Hawaii beagle	28 Feb-24 Mar 72	7	passed at approx. weekly intervals
6-19	PDK	II. Hawaii beagle	10 Apr-30 Nov 72	7	passed at approx. weekly intervals
20-45	PDK	230 Dow Chem.	3 Mar- 6 Dec 73	7	passed at approx. weekly intervals
46-50	PDK	230 Dow Chem.	13 Dec 73-14 Feb 74	7	passed at approx. weekly intervals
GMK					
1-5	GMK	I. Hawaii AGM 1	6 Mar- 3 Apr 72	7	passed at approx. weekly intervals
6-12	GMK	II. Hawaii AGM 1	3 May-26 Sep 72	7	passed at approx. weekly intervals
13-15	GMK	I. Hawaii AGM 1	3 Oct-17 Oct 72	7	passed at approx. weekly intervals
16-19	GMK	II. Hawaii AGM 1	24 Oct-14 Nov 72	7	passed at approx. weekly intervals
20-30	GMK	III. Hawaii AGM 2	28 Nov 72-15 Apr 73	7	passed at approx. weely intervals
31-50	GMK	VI. Hawaii AGM 4	28 Apr-13 Aug 73	7	passed at approx. weekly intervals

Table 2. Summary of biological properties of DEN 4 (H-241) at different passage levels in PDK cell cultures

		LLC-MK2	PDK (Uncloned)			
	Biological attribute	parent	7	15	30	50
In vitro	ts 39°C	0	0	0	0	+
	GMK plaques	+	0	0	0	0
	LLC-MK2 plaques*	Large	Medium	Small	Pinpoint	Pinpoi
	LLC-MK2 CPE	+	0	0	0	0
	Growth in human monocytes	+	0	0	0	0
In vivo	Monkey viremia	14/14	2/2	2/4	0/4	0/4
	HI antibody response	14/14	2/2	4/4	4/4	2/4
	PRNT antibody response	14/14	2/2	4/4	2/4	2/4
	2° response to parent challenge	—	—	2/4	4/4	4/4
	Monkey neurovirulence	0/1	ND	0/1	0/1	0/1
	Suckling mouse i.c. neurovirulence ave. survival time (days)	11.0	13.1	14.0	15.8	16.3
	Growth in Aedes albopictus intrathoracic inoc.	+	ND	+	+	+

* Plaque size: Large = ≥5 mm
Medium = 2-4 mm
Small = 1 mm
Pinpoint = <1 mm

Table 3. Summary of biological properties of DEN 4 (H-241) at different passage levels in GMK cell cultures

		LLC-MK2	GMK (Uncloned)			
	Biological attribute	parent	7	15	30	50
In vitro	ts 39°C	0	ND	0	0	0
	GMK plaques	+	ND	0	0	0
	LLC-MK2 plaques*	Large	Medium	Medium	Small/ Large	Small/ Large
	LLC-MK2 CPE	+	0	0	0	0
	Growth in human monocytes	+	ND	0	0	0
In vivo	Monkey viremia	14/14	2/2	0/2	1/2	1/2
	HI antibody response	14/14	2/2	2/2	2/2	2/2
	PRNT antibody response	14/14	2/2	2/2	2/2	2/2
	2° response to parent challenge	—	--	--	--	--
	Monkey neurovirulence	0/1	ND	ND	ND	ND
	Suckling mouse i.c. neurovirulence ave. survival time (days)	11.0	ND	11.5	12.0	11.7
	Growth in Aedes albopictus intrathoracic inoc.	+	+	+	+	+

* Plaque size (see Table 2)

Table 4. Growth of DEN-4 viruses following intrathoracic inoculation of adult Aedes albopictus. Virus content of mosquitoes was assayed after 10 days incubation at 28°C

Virus Strain	No. mosq. inoc.	Total PFU Inoc./mosq.	Mosq. susp. titer pfu/ml	Mosq. susp. Vol (ml)	Total PFU/mosq.
parent	29	170.0	1.8×10^4	15	2.7×10^5
GMK 7	12	5.1	1.2×10^4	6	7.2×10^4
GMK 15	24	15.3	3.5×10^4	13	4.6×10^5
GMK 30	28	130.0	3.8×10^4	14	5.3×10^5
GMK 50	10	50.3	2.7×10^4	5	1.4×10^5
PDK 7	30	0.14	1.8×10^4	15	2.7×10^5
PDK 15	24	0.85	2.5×10^4	13	3.2×10^5
PDK 30	22	95.0	2.2×10^4	11	2.4×10^5
PDK 50	10	30.7	1.3×10^3	5	6.5×10^3

Table 5. Examples of HI and neutralizing antibody responses to challenge with parental DEN 4 in monkeys previously immunized with PDK passaged virus. All animals challenged 42 days after initial infection

Monkey No.	Initial Infecting Strain	Day After 2nd Infection	Viremia	Reciprocal HI Titer DEN 1	DEN 2	DEN 3	DEN 4	Reciprocal DEN 4 PRNT 1	Ig Isotype	Comment
H-147	GMK 15	0	0	10	10	20	160	480		
		5		10	20	20	160			
		10		10	10	10	320		IgG	solid
		14	0	10	10	10	320			protection
		21		10	10	10	160			
		42		20	20	20	80	560		
H-177	PDK 30	0	0	<10	<10	<10	10	80		
		5		<10	<10	<10	20			secondary
		10		10	10	<10	80		IgG	type
		14	0	10	20	<10	160			response
		21		10	20	<10	80			
		42		10	20	<10	40	320		
H-246	PDK 50	0	D1,D2	<10	<10	<10	<10	<10		
		5	0	<10	<10	<10	10			primary
		10		10	10	20	80		IgM/	immunization
		14	0	80	40	80	640		IgG	failure
		21		40	40	40	320			
		42		40	80	40	640	400		

Table 6. Days of viremia and HI antibody titer on day 42 following inoculation of susceptible rhesus monkeys with passaged DEN 4 viruses. Each entry represents an individual monkey.

	PDK Series			GMK Series		
		Reciprocal			Reciprocal	
Passage level	Monkey No.	DEN 4 HI titer Day 42	Days of Viremia*	Monkey No.	DEN 4 HI titer Day 42	Days of Viremia
parent	H-155	320	2,3,4 LP			
parent	H-187	640	2,3,4 LP			
parent	H-228	160	1,2,3,4 LP			
parent	H-224	320	1,2,3,4 LP			
7	H-158	160	5,7 MP	H-148	160	3,4,5,6 LP
15	H-157	160	7 MP	H-147	160	none
15	H-180	20	none	H-182	10	none
15	H-235	320	7,8,9 MP			
15	H-236	30	4,6-9 MP			
30	H-177	<10	none	H-179	320	7,8,9,10 LP
30	H-161	10	none	H-185	320	none
30	H-233	10	none			
30	H-249	<10	none			
50	H-198	<10	none	H-199	<10	none
50	H-245	40	none	H-201	160	6,7,8 LP
50	H-246	<10	none			
50	H-250	<10	none			

* Plaque size (see Table 2)

Table 7. Days of viremia and selected HI antibody titers in rhesus monkeys inoculated with PDK or GMK passaged virus and then challenged with parental DEN 4.

	PDK Series				GMK Series			
Passage level	Monkey No.	Challenge dose	Days of viremia	Reciprocal HI titer Day 14	Monkey No.	Challenge dose	Days of viremia	Reciproca HI titer Day 14
parent	H-155	2×10^4	0	640				
parent	H-187	2×10^4	0	320				
parent	H-228	6×10^4	0	640				
parent	H-224	6×10^4	0	320				
7	H-158	2×10^4	0	640	H-148		0	1280
15	H-157	2×10^4	0	320	H-147		0	320
15	H-180	2×10^4	0	320	H-182		0	320
15	H-235	6×10^5	0	320				
15	H-236	6×10^5	0	80				
30	H-177	2×10^4	0	160	H-179		0	320
30	H-181	2×10^4	0	640	H-185		0	160
30	H-233	6×10^5	1,2,3	640				
30	H-249	6×10^5	0	80				
50	H-199	2×10^4	2,3	640*	H-198		0	640
50	H-245	6×10^5	0	80*	H-201			
50	H-246	6×10^5	1,2	640				
50	H-250	6×10^5	1,4	160*				

* Secondary-type antibody responses evidenced by early (day 8) IgG antibody response

Table 8. Summary of biological properties of DEN 4 (H-241) PDK-passaged viruses cloned three times using 10-fold dilutions

		LLC-MK2	PDK		
	Biological attribute	parent	19-3C1	34-3C1	56-3C1
In vitro	ts 38.5°C	0	+	+	+
	GMK plaques	+	0	0	0
	LLC-MK2 plaques	9-12 mm sharp	2 mm hazy	2 mm hazy	1 mm sharp
	LLC-MK2 CPE	+	0	0	0
	Growth in human monocytes	+	0	0	0
	Monkey viremia	14/14	0/6	5/6	0/6
In vivo	HI antibody response	14/14	3/6	5/6	3/6
	PRNT antibody response	14/14	3/6	5/6	3/6
	2° response to parent challenge	—	3/6	0/6	2/6
	Breakthrough viremia	—	5/6	1/6	5/6
	Suckling mouse i.c. neurovirulence ave. survival time (days)	7.8	10.7	11.5	10.8

Table 9. Average survival time of 1-2 day old mice inoculated with parental DEN 4 and different cloned PDK passaged virus strains. All mice inoculated with approximately 100 pfu intracerebrally.

Virus	Days of observation / Cumulative deaths/total											Average survival time	Mouse brain titer	
Strain	0	7	8	9	10	11	12	13	14	15	18	(days)	(pfu/ml)	Observations
Parent	0/14	2/14	14/14									7.8	1×10^4	LP
19-3C1	0/12	0/12	0/12	0/12	4/12	12/12						10.7	1×10^7	SP,LP
34-3C1	0/12	0/12	0/12	0/12	0/12	6/12	12/12					11.5	5×10^6	SP,LP
56-3C1	0/12	0/12	0/12	0/12	2/12	12/12						10.8	4×10^5	SP,LP
Parent	0/16	0/16	0/16	0/16	0/16	0/16	16/16					12.0	8×10^4	LP
24-TD3							2/16	8/16	16/16			13.9	2×10^7	SP
35-TD3							5/16	5/16	5/16	6/16	16/16	15.9	3×10^6	SP
61-TD3							5/16	10/16	10/16	10/16		13.4	3×10^7	Pinpoint

Table 10. Growth of dengue 4 strains in human monocytes.

DEN-4 strains	MOI	Day 4 pfu/ml
parental	0.1	415
19-3C1	0.05	<16
34-3C1	0.1	<16
56-3C1	0.1	<16
25-TD3	0.1	<16
36-TD3	0.09	<16
62-TD3	0.1	<16

Table 11. Days of viremia and selected HI antibody titers in rhesus monkeys following inoculation of FDK-3Cl strains and challenge with parental DEN 4 virus

			Primary Immunization		Parental DEN-4 (H-241) Mk2p3 challenge (3.6×10^6 pfu inoc)	
Monkey No.	Virus Strain	Virus Dose (pfu)	Days of Viremia	Reciprocal DEN 4 HI Titer (PRNT)	Days of Viremia	Reciprocal DEN 4 HI Titer (Day 14)
R-172	19-3Cl	4×10^2	0	10	1,2	640
R-189	19-3Cl	4×10^2	0	<10	1,2,3	640
R-204	19-3Cl	1.7×10^3	0	40	2	80
R-205	19-3Cl	1.7×10^3	0	<10	2	2560
R-212	19-3Cl	8×10^2	0	<10	1,4,6	320
R-213	19-3Cl	8×10^2	0	20	1,2	640
R-190	34-3Cl	4×10^2	6,7,9,11(L)	1280	0	320
R-191	34-3Cl	4×10^2	0	<10	6-12	<10
R-206	34-3Cl	2×10^2	4,5,6 (L)	160	0	160
R-208	34-3Cl	2×10^2	5,6 (L)	320	0	320
R-215	34-3Cl	1.6×10^4	6-10 (L)	320	0	320
R-215	34-3Cl	1.6×10^4	5,6,7 (L)	160	0	160
R-195	56-3Cl	1.2×10^4	0	10	1,2,3,5	640
R-196	56-3Cl	1.2×10^4	0	<10	3,4	640
R-209	56-3Cl	1×10^4	0	10	1,2,3,4	640
R-210	56-3Cl	1×10^4	0	<10	1,2,4	5120
R-216	56-3Cl	1.6×10^3	0	<10	2,5	320
R-217	56-3Cl	1.6×10^3	0	80	0	40

* All antibody shown was of the IgG class

Table 12. Summary of biological properties of DEN 4 (H-241) PDK-passaged viruses cloned three times using 2-fold dilutions.

	Biological attribute	LLC-MK2 parent	24-TD3	35-TD3	61-TD3
In vitro	ts 38.5°C	0	+	+	+
	GMK plaques	+	0	0	0
	LLC-MK2 plaques	7-9 mm	1-2 mm	1-2 mm	1 mm
	LLC-MK2 CPE	+	0	0	0
	Growth in human monocytes	+	0	0	0
	Monkey viremia	14/14	0/6	0/6	2/5
	HI antibody response	14/14	1/6	1/6	5/5
In vivo	2° response to parent challenge	—	6/6	5/6	1/5
	Challenge virus Breakthrough viremia	0/14	6/6	5/6	1/5
	Suckling mouse i.c. neurovirulence ave. survival time (days)	12.0	16.7	17.2	15.3

Table 13. Days of viremia and selected HI antibody titers in rhesus monkeys following inoculation of PDK-TD3 virus strains and challenge DEN 4 virus.

			Primary Immunization		Parental DEN 4 challenge (6×10^4 pfu)	
				Day 42		Reciprocal
Monkey No.	Virus Strain	Virus Dose (pfu)	Days of Viremia	Reciprocal DEN 4 HI Titer (PRNT)	Days of Viremia	DEN 4 HI titer (Day 14)
H-226	24-TD3	3×10^3	0	<10 (10)	1	320
H-229	24-TD3	3×10^3	0	<10 (<10)	1,2	320
H-238	24-TD3	2.5×10^4	0	<10 (<10)	1	320
H-239	24-TD3	2.5×10^4	0	10 (<10)	1,4	320
H-250	24-TD3	7×10^3	0	<10 (<10)	1	320
H-252	24-TD3	7×10^3	0	<10 (<10)	1	320
H-231	35-TD3	3×10^4	0	<10 (<10)	1	320
H-230	35-TD3	3×10^4	0	<10 (<10)	1	320
H-240	35-TD3	1.4×10^5	0	<10 (<10)	1	>640
H-241	35-TD3	1.4×10^5	0	10 (10)	0	>640
H-253	35-TD3	4×10^4	0	<10 (<10)	1	320
H-254	35-TD3	4×10^4	0	<10 (<10)	1	320
H-232	61-TD3	1.3×10^5	9 L*	80 (780)	0	80
H-227	61-TD3	1.3×10^5	0	<10 (12)	1	640
H-242	61-TD3	2.3×10^5	9,10,11,12 L*	320 (160)	0	320
H-243	61-TD3	2.3×10^5	0	80 (20)	0	160
H-244	61-TD3	2.3×10^5	0	40 (54)	0	80

* Temperature resistant

Table 14. Effect on plaque size and temperature sensitivity on DEN-4 TD3 viruses serially passaged in LLC-MK2 cells at 7 day intervals.

Virus Strain	Titer (PFU/ml)	Plaque Size*	$ts_{38.5}$
PDK 24-TD3 LLC-MK2 p1	$1.? \times 10^7$	PP	+
p2	1.3×10^4	SP	+
p3	1.2×10^5	MP	+
PDK 35-TD3 LLC-MK2 p1	1.2×10^6	PP	+
p2	2.1×10^3	SP	+
p3	1.8×10^4	MP	+
PDK 61-TD3 LLC-MK2 p1	1.8×10^7	PP	+
p2	1.3×10^3	SP	+
p3	6.0×10^3	MP	+

* Plaque size after 7 days incubation at room temperature

PP = pinpoint

SP = 1 mm

MP = 2-4 mm

Table 15. Effect on plaque size and temperature sensitivity of serial passage of DEN-4 strains in FRhL cells. Passage was done at 35°C

FRhL Passage No.	Titer (pfu/ml)	PDK 24-TD3 Plaque Size[1]	$ts_{38.5}$
1	6×10^4	PP	+
2	1×10^3	PP	
3	6×10^3	PP	
4	2×10^5	PP	
5	1×10^6	PP/SP	
6	3×10^5	PP/SP	
7	1×10^5	PP/SP	
8	3×10^4	SP	
9	8×10^4	SP	
10	1×10^5	SP	±*

* pfu/ml $\frac{38.5^{\circ}C}{37^{\circ}C} = \frac{1 \times 10^2}{8 \times 10^4}$

[1] Plaque size:

PP = pinpoint

SP = 1 mm

MP = 2-4 mm

Table 16. Effect on plaque size, temperature sensitivity and monkey virulence of prolonged replication of PDK 24-TD3 virus in FRhL cells. Incubation temperature 35°C. Input dose 4×10^4 pfu/8 ounce bottle.

Day After Infection	$MgSO_4$ 40 mM	XCV pfu/ml	Plaque Size	$ts_{38.5}$	Parental-type Viremia in Monkeys
8		6×10^1	SP	+	
9		4×10^1	SP	+	
12	+	4×10^3	SP	+	
13	+	1×10^2	SP	+	
15	+	2×10^2	SP, 1 LP	0	+
16	+	7×10^7	SP	+	
17	+	5×10^2	SP	+	
19		2×10^2	SP,LP	0	+
19 (CAV*)		3×10^5	SP,LP	0	+

XCV = Extracellular Virus

CAV = Cell-Associated Virus

Table 17. Effect on plaque size, temperature sensitivity and monkey virulence of prolonged replication of PDK 24-TD3 virus in LLC-MK2 cells. Incubation temperature 37°C. Input dose 5×10^4 pfu/8 ounce bottle.

Day after infection	Titer (pfu/ml)	Plaque Size	$ts_{38.5}$	Parental-type Viremia in Monkeys
8	3×10^3	MP	+	None
9	4×10^4	MP	+	
10	3×10^4	MP	+	
11	2×10^3	MP	+	
12	5×10^3	MP	+	
13	1×10^3	MP	+	
14	5×10^3	MP	+	
15	3×10^4	MP	+	
16	5×10^4	MP	+	None

Table 18. Effect on plaque size of the phenotype and dose of PDK-TD3, FRhL p2 inoculated in FRhL cells. Incubation temperature 32°C. Virus harvested in MM with 70 mM $MgSO_4$.

Inoculum Dose (pfu/ml)	Plaque Size	Day of Harvest	Titer (pfu/ml)	Plaque Size
4	SP	5	3×10^1	SP
		6	6×10^1	SP
		7	5×10^2	SP
		8	2×10^3	SP, 1 MP
1.7×10^2	SP	5	6×10^3	SP, 1 MP
		6	1×10^4	SP
		7	4×10^4	SP, LP
		8	2×10^4	SP
2.2×10^3	SP & LP	5	5×10^4	SP
		6	8×10^4	SP
		7	9×10^4	SP, LP
		8	1×10^5	SP
1.4×10^4	SP & LP	5	3×10^5	SP, MP
		6	2×10^5	SP, LP
		7	2×10^5	SP, MP
		8	5×10^4	SP, LP

Table 19. Effect on plaque size of the phenotype and dose of PDK 24-TD3 FRhL p2 inoculated in primary dog kidney cells. Incubation temperature 32°C. Virus harvested in 70 mM $MgSO_4$.

Inoculum		Harvest on Day 9	
Dose (pfu/ml)	Plaque Size	Titer (pfu/ml)	Plaque Size
7	SP	2.5×10^3	SP
12	LP	2×10^2	SP
7×10^1	SP	4×10^2	SP
2×10^3	SP, LP	3×10^2	SP

Table 20. Effect on plaque size of passaging small plaque PDK 24-TD3, FRhL p2, PDK p1 virus in FRhL cells. Incubation temperature 32°C.

Inoculum		Passage in FRhL		
Dose (pfu/ml)	Plaque Size	Day of Harvest	Titer (pfu/ml)	Plaque Size
3×10^3	SP	4	4×10^2	SP, MP
		5	2×10^3	SP, MP
		6	2×10^3	SP, MP
		7	2×10^3	SP, MP
		8	1×10^3	SP, MP*

* This strain was temperature resistant

Table 21. Effect on plaque size and temperature sensitivity of ten serial passages of PDK 35-TD3 in FRhL cells. Incubation temperature was 37°C. Cells plus extracellular fluid harvested after 7 days incubation.

FRhL Passage No.	Titer (pfu/ml)	Plaque Size	$ts_{38.5}$	Parental-type Viremia in Monkeys
1	8×10^3	PP	+	
2	1×10^2	PP		
3	5×10^1	PP		
4	<6.5*	—		
5	<6.5	—		
6	<6.5	—		
7	<6.5	—		
8	1×10^2	SP		
9	8×10^3	SP/MP		
10	1×10^5	SP/MP	+**	0*

* Medium plaque pick:

$$\text{pfu/ml at } \frac{38.5^\circ C}{37^\circ C} = \frac{0}{2 \times 10^5}$$

** No viremia in rhesus H-262 following inoculation of 3×10^3 pfu

Table 22. Effect on plaque size and temperature sensitivity of serial passage of PDK 35-TD3 in FRhL cells. Extracellular fluid harvested in 70 mM $MgSO_4$ on days 7-10. Incubation temperature 32°C.

			FRhL passage Dose (pfu/ml) Plaque Size Day of Harvest							
Dose (pfu/ml)	Plaque Size	Passage No.	7		8		9		10	
2.0×10^4	SP	1	3×10^4	SP	9×10^3	SP/MP	7×10^3	SP/MP	3×10^3	SP
6.8×10^3	SP	2	2×10^3	SP	1×10^4	SP	9×10^3	SP/MP	3×10^4	SP/MP
3.6×10^2	SP	3	2×10^4	SP	9×10^3	SP	3×10^4	SP	2×10^4	SP
4.4×10^3	SP	4	1×10^3	SP	2×10^3	SP/MP	2×10^3	SP	3×10^2	SP
2.0×10^2	SP	5	3×10^2	SP	7×10^2	SP/MP	5×10^2	SP	8×10^2	SP

Table 23. Selected Viremia and HI antibody responses in susceptible rhesus monkeys inoculated with mixed small, medium and large plaque virus or homogeneous medium and large plaque virus derived from PDK 35-TD3 replicated in FRhL cells.

	Plaque Picked PDK 35-TD3 Virus Strains								
	FRhL p1 (SP/MP) viremia			FRhL p1 (LP) viremia			FRhL p2 (SP/MP) viremia		
Day after Inoculation	Direct	Delayed	HI	Direct	Delayed	HI	Direct	Delayed	HI
1	<16.5	<16.5	<10	<16.5	<16.5	<10	<16.5	<16.5	
2									
3								SP/MP	
4								<16.5	
5								SP/MP	
6							<16.5	<16.5	
7		<16.5					16.5 LP	MP	
8		SP/MP					<16.5	SP/MP	
9		SP/MP						<16.5	
10		LP							
11		<16.5							
12	<16.5	<16.5	<10	<16.5	<16.5		<16.5	<16.5	<10
14			10			<10			40
21			80			40			80
28			40			20			80
42			40			40			--

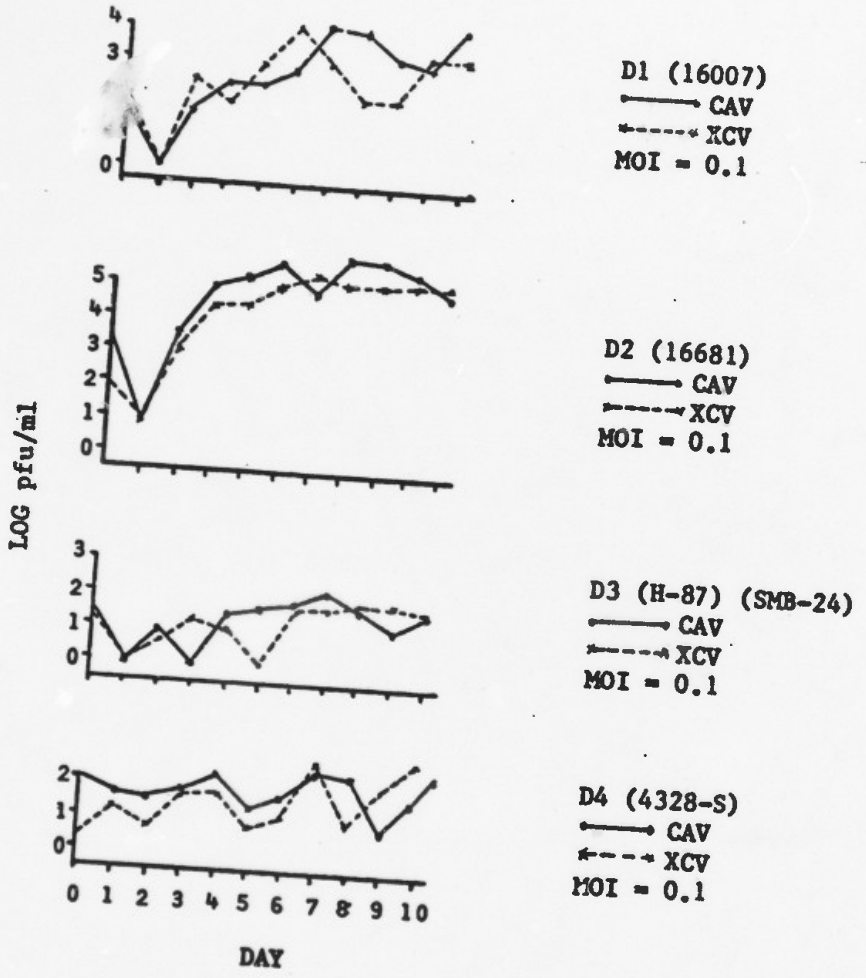
Table 24. Summary of biological properties of PDK 35-TD3 dengue 4 (H-241) Production Seed and Candidate Vaccine.

		LLC-MK2	PDK 35-TD3	
	Biologic Attribute	parent	FRhL p2	FRhL p3
In vitro	ts 38.5°C	0	+	+
	GMK plaques	+	0	0
	LLC-MK2 plaques	LP	SP,MP	SP,MP
	LLC-MK2 CPE	+	0	0
	Growth in human monocytes	+	0	0
In vivo	Monkey viremia	14/14	1/4	2/4
	HI antibody response	14/14	3/4	4/4
	PRNT antibody response	14/14	2/4	4/4
	2° response to parent challenge	—	2/4	—
	Monkey viremia	0/3	0/17	ND
	Suckling mouse average survival time (days	11.0	15.5	16.9

Table 25. Days of viremia and selected antibody responses in rhesus monkeys following inoculation of DEN 4 PDK 35-TD3 Production Seed (FRhL p2) and Candidate Vaccine (FRhL p3).

Monkey No.	Virus Strain	Virus Dose (pfu/ml)	Primary Immunization		Parental Challenge	
			Days of Viremia	Reciprocal DEN 4 antibody (Day 42)	Days of Viremia	Reciprocal DEN 4 HI titer (Day 14)
UH-25	FRhL p2	8×10^3	12, MP	640 (10)	0	640
UH-31			0	<10 <10	1, LP	640
UH-38			C	40 10	0	80
UH-40			0	10 <10	3, LP	640
18082	FRhL p3	1×10^4	7-12, MP	40 680	0	80
UH-41			0	40 31	0	40
UH-37			8,12-14,MP	80 305	0	80
18192			8-12, MP	80 160	0	40

Figure 1. Growth of D1-4 viruses in primary dog kidney cells at 32°C.



CAV = Cell Associated Virus

XCV = Extracellular Virus

Figure 4. Terminal dilution diagram

Strain	Reciprocal Dilution	1	2	3	4	5	6	7	8	9	10
PDK 19-3C1	16,000	0	+	0	+	+	0	0	0	0	0
	32,000	⊕	0	0	0	0	0	0	0	0	0
PDK 20 (TD-1) 13	8	+	0	+	+	+	0	0	0	0	0
	16	0	0	0	0	0	0	0	0	0	0
	32	0	+	0	0	0	0	0	0	0	0
PDK 21 (TD-2) 108	1280	0	+	+	0	0	+	0	0	0	0
	2560	0	0	0	0	0	0	0	0	⊕	0
PDK 22 (TD-3) 100	PDK 24 TD3 plaque size: predominant small (1 mm), occasional medium (2 mm).										
PDK 30	5×10^{-5}	0	0	0	0	0	+	+	0	0	0
	10^{-6}	0	0	0	0	0	⊕	0	0	0	0
PDK 31 (TD-1) 425	8,000	+	0	+	+	+	+	0	+	0	0
	16,000	⊕	0	0	0	0	0	0	0	0	0
PDK 32 TD-2 100	1280	+	+	+	0	0	0	0	- 0	0	0
	2560	0	0	0	0	0	+	0	0	0	0
	5120	⊕	0	0	0	0	0	0	0	0	0
PDK 33 TD-3 45	PDK 35 TD3 plaque size: small (1 mm).										
PDK 56 3C1	2.5×10^5	0	+	0	0	0	0	0	0	0	0
	5.1×10^5	0	0	0	0	⊕	0	0	0	0	0
PDK 57 TD-1 204	320	0	0	0	0	+	+	0	0	0	0
	640	0	0	0	0	0	⊕	0	0	0	0
PDK 58 TD-2 6000	5120	0	0	0	0	0	+	0	0	+	0
	10240	0	0	0	0	0	⊕	0	0	0	0
PDK 59 TD-3 238	PDK 61 TD3 plaque size: small (0.5 mm).										

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